ELICITATION EFFECT ON THE GROWTH AND BIOCHEMICAL ACTIVITIES OF *CURCUMA MANGGA* VAL (ZINGIBERACEAE) *IN VITRO* PLANTLETS

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by

FARIZ ABRAHAM

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"Commit to the LORD whatever you do, and your plans will succeed."

Proverbs of King Solomon

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ºC	Degree Celcius
ANOVA	Analysis of Variance
B	Boron
BAP	6-Benzylaminopurine
BHT	Butyl Hydroxy Toluene
cm	Centimetres
Co	Cobalt
Cu	Copper
DMSO	Dimethyl sulphoxide
DPPH	2,2-diphenyl-1-picrylhydrazyl
e^-	Electron
EC_{50}	Effective minimum concentration to inhibit by 50%
EDTA	Ethylene-diamine-tetraaceticacid
ET	Electron Transfer
FAA	Formalin- Acetic acid- Alcohol
FCR	Folin- Ciocalteu Reagent
Fe	Ferrum (Iron)
FTC	Ferric Thiocyannate
GGPP	Geranyl-geranyl phyrophosphate
HAT	Hydrogen Atom Transfer
HSD	Honestly Significant Difference

IAA	Indole-3-acetic acid
L-DOPA	$_{L}$-3,4-dihydroxyphenylalanine or Levodopa
LED	Light Emitting Diode
MCF7	Michigan Cancer Foundation 7
mg	milligram
mg/ml	milligram per millilitre
mgL^{-1}	Milligram per litre
min	minutes
ml	millilitre
Mn	Manganese
Mo	Molybdenum
MS	Murashige and Skoog
NAA	1-Naphthaleneacetic acid
OVCAR3	Human ovarian cancer line 3
pH	A measure of acidity or alkalinity of solution
RCBD	Randomized Complete Block Design
RSA	Free Radical Scavenging Activity
SE	Standard Error
TBA	Tetra Butyl Toluene
TCM	Traditional Chinese medicine
µgGAE/mg extract	microgram gallic acid equivalent per milligram extract
µgmL	microgram per millilitre
µM	micromolar
$\mu mol\ m^2\ s^{-1}$	Micromole per meter square in one second
$^{v}/_{v}$	Volume (ml) per volume (ml)

$^{w}/_{v}$	Weight (g) per volume (ml)
YE	Yeast extract
Zn	Zinc

KESAN ELISITASI TERHADAP PERTUMBUHAN DAN AKTIVITI BIOKIMIA ANAK BENIH *IN VITRO CURCUMA MANGGA* VAL. (ZINGIBERACEAE)

ABSTRAK

Anak benih aseptik *Curcuma mangga* boleh dihasilkan melalui teknik pensterilan permukaan berganda. Eksplan tunas daripada rizom direndamkan di dalam 70% ($^{v}/_{v}$) alkohol selama 10 minit pada peringkat pertama diikuti dengan pensterilan permukaan menggunakan 20% ($^{v}/_{v}$) Clorox® selama 20 minit untuk peringkat kedua. Eksplan aeptik kemudian dikulturkan ke dalam medium proliferasi yang terbaik iaitu, medium MS yang mengandungi 2 mgL^{-1} 6-benzylaminopurine (BAP) dan 0.5 mgL^{-1} 1-naphthaleneacetic acid (NAA). Medium proliferasi cecair merangsangkan lebih pucuk aksil (6.7 ± 0.4 pucuk/eksplan) berbanding dengan medium proliferasi pepejal (4.6 ± 0.6 pucuk/eksplan). Suku bahagian pucuk eksplan menghasilkan lebih banyak pucuk (7.6 pucuk/ eksplan) berbanding dengan yang dibahagikan separuh dan seluruh pucuk eksplan. Mod inokulasi (menegak atau mendatar) tidak mempengaruhi pengahasilan pucuk of *C. mangga*. pH medium yang berbeza (5.7, 7.0 and 8.0), keadaan pencahayaan (pencahayaan berterusan and keadaan gelap), dan kepekatan ekstrak yis (0-5.0 mgL^{-1}) yang ditambahkan ke dalam medium kultur tidak mempengaruhi biosijim anak benih dan bilangan pucuk baru yang dihasilkan daripada setiap eksplan. Kerencatan pertumbuhan dan klorosis seringkali didapati pada anak pokok di dalam medium proliferasi alkali (pH 8.0), kepekatan ekstrak yis yang tinggi (3.5 and 5.0 mgL^{-1}), kepekatan chitosan yang

tinggi (100-200 mgL^{-1}), dan tanpa atau kekuatan mikronutrien yang lebih tinggi daripada kandungan normal micronutrien dalam medium MS (0 atau 3-12 kekuatan MS mikronutrien). Pertumbuhan etiolasi dan klorosis jelas sekali didapati pada anak benih yang dikulturkan dalam keadaan gelap. Perubahan abnormal yang lain seperti kerapuhan petiol dan hambatan pembentukan pucuk dan akar hanya boleh dikesan pada anak pokok yang dikultur pada medium yang mengandungi kepekatan tinggi chitosan (100-200 mgL^{-1}) + 3.5 mgL^{-1} ekstrak yis. Kehadiran chitosan dalam kepekatan tinggi (150 mgL^{-1}) + 3.5 mgL^{-1} ekstrak yis secara signifikan mempengaruhi penghasilan pucuk baru berbanding dengan kawalan. Anak benih *C. mangga* hanya boleh bertoleransi dengan kehadiran separuh dan sepenuh kandungan kekuatan medium MS mikronutrien. Biosijim anak benih (segar dan kering) dipengaruhi oleh kehadiran MS mikronutrien lebih tinggi daripada kekuatan penuh atau tanpa kehadiran MS mikronutrien tetapi tidak mempengaruhi penghasilan pucuk baru. Anak benih yang dikulturkan di dalam medium yang mengandungi ekstrak yis (3.5 dan 5.0 mgL^{-1}) dan di dalam medium yang mengandungi 150 mgL^{-1} chitosan + 3.5 mgL^{-1} ekstrak yis menunjukan nilai aktiviti penyingkiran radikal bebabs yang tinggi dan stabil dari 30 hingga 130 minit semasa waktu dieramkan. Kompaun fenolik ialah bukan factor yang utama daripada anak benih *C. mangga* bagi aktiviti penyingkiran radikal bebas. Ini dibuktikan dengan korelasi yang rendah di antara jumlah keseluruhan kandungan fenolik (TPC) dan aktiviti penyingkiran radikal bebas (RSA) oleh ekstrak anak benih *C. mangga*. Ekstrak yis mungkin merangsang penghasilan kompaun antioksidan bukan fenolik sepertimana yang ditunjukan oleh korelasi yang baik di antara biosijim dan aktiviti penyingkiran radikal bebas (0.8863). Ekstrak mentah asal daripada anak benih yang dikulturkan dalam medium yang mengandungi 3.5 and 5.0 mgL^{-1} ekstrak yis dan dalam medium yang mengandungi

150 mgL^{-1} khitosan + 3.5 mgL^{-1} ekstrak yis merencat lebih kurang 30% peperoksidaan lipid di mana ia menunjukan aktiviti yang sama seperti kuersetin.

ELICITATION EFFECT ON THE GROWTH AND BIOCHEMICAL ACTIVITIES OF *CURCUMA MANGGA* VAL. (ZINGIBERACEAE) *IN VITRO* PLANTLETS

ABSTRACT

Curcuma mangga aseptic plantlets could be established via double-stage surface sterilization technique. The bud explants derived from the rhizomes were immersed in 70% ($^{v}/_{v}$) alcohol for ten minutes at the first stage followed by surface-sterilized with 20% ($^{v}/_{v}$) Clorox® for 20 minutes at the second stage. The established aseptic explants were then cultured on the best proliferation medium, which is the MS medium supplemented with 2 mgL^{-1} 6-benzylaminopurine (BAP) and 0.5 mgL^{-1} 1-naphthaleneacetic acid (NAA). Liquid proliferation medium induced more auxiliary shoots (6.7 ± 0.4 shoots/explant) as compared to gelled proliferation medium (4.6 ± 0.6 shoots/explant). Quarterly divided shoot explants produced more shoots (7.6 shoots/ explant) compared to half divided and whole shoot explants. Mode of inoculation (vertically or horizontally) did not affect shoot production of *C. mangga*. Different pH of the culture medium (5.7, 7.0 and 8.0), illumination conditions (continuous illumination and total darkness), and concentration of yeast extract (0-5.0 mgL^{-1}) added into the culture medium did not affect the plantlet biomass and number of new shoots produced from each explants. Growth retardation and chlorosis were often detected in plantlets cultured in alkaline proliferation medium (pH 8.0), high concentration of yeast extract (3.5 and 5.0 mgL^{-1}), high concentration of chitosan (100-200 mgL^{-1}), and the absence or higher strength than normal MS micronutrients strength (0 or 3-12 strength of MS micronutrients).

Etiolated growth and chlorosis were obviously detected in plantlets cultured in total darkness condition. Other abnormal changes such as brittle petiole and inhibition of shoots and roots formation were only detected in plantlets cultured in medium supplemented with high concentration of chitosan (100-200 mgL^{-1}) + 3.5 mgL^{-1} yeast extract. The presence of high concentration of chitosan (150 mgL^{-1}) + 3.5 mgL^{-1} yeast extract significantly affected the new shoots production as compared to control. *C. mangga* plantlets could only tolerate the presence of half and full strength MS micronutrients in medium. Biomass (fresh and dried) of plantlets was affected by the presence of MS micronutrients higher than full strength or without the addition of MS micronutrients in medium but not the number of new shoots produced. Plantlets cultured in medium supplemented with yeast extract (3.5 and 5.0 mgL^{-1}) and in medium supplemented with 150 mgL^{-1} chitosan + 3.5 mgL^{-1} yeast extract exhibited high and stable free radical scavenging activity from 30 to 130 minutes of incubation time. Phenolic compounds were not the major contributor of free radical scavenging activity of *C. mangga* plantlets. It was proven by the low correlation between total phenolic content (TPC) and free radical scavenging activity (RSA) of the plantlets. Yeast extract might induce the production of non phenolic antioxidant compounds as it was represented by a good correlation between biomass and radical scavenging activity (0.8863). The crude extract derived from plantlets cultured in medium supplemented with 3.5 and 5.0 mgL^{-1} yeast extract and in medium supplemented with 150 mgL^{-1} chitosan + 3.5 mgL^{-1} yeast extract inhibited 30% of lipid peroxidation which was similar to quercetin.

CHAPTER ONE

INTRODUCTION

Zingiberaceae is a well-known ginger family in Southeast Asia and many of its species are being used in traditional medicine for treatment of several diseases. Rhizomes of Zingiberaceae plant species have been widely used as spices or condiments. Rhizomes are often eaten raw or cooked as vegetables and used for flavouring food. The rhizomes of ginger plants are commonly consumed by women during confinement. They are also taken as carminatives for relieving flatulence. Commercially cultivated Zingiberaceae species are *Zingiber officinale*, *Curcuma longa*, and *Alpinia galanga*. *Curcuma mangga*, another Zingiberaceae species, is a popular vegetable, of which the tips of young rhizomes and shoots are consumed raw with rice. Traditionally, the rhizomes are used for stomach ache, chest pains, fever, and general debility (Larsen *et al*., 1999). Recently, mango ginger (*C. mangga*) and white turmeric (*C. zedoaria*) rhizomes have been utilized to treat cancer although clinically their activity has not been tested. The cytotoxicity of *C. mangga* and *C. zedoaria* rhizomes extracts (chloroform and methanol) had been tested on human cancer cell lines and all of the extracts exhibited very low cytotoxic activity compared to the positive control (doxorubicin and cisplatin) (Hartati *et al*., 2003). Zerumin B, a compound isolated from *C. mangga* rhizome, exhibited potent activity against human breast cancer line (MCF7) (Abas *et al*., 2005b). Many studies on antioxidant properties of ginger species were confined to rhizomes (Habsah *et al.*, 2000; Jitoe *et al.*, 1992; Zaeoung *et al.*, 2005).

Leaves of ginger plants have also been used for food flavouring and in traditional medicine. In Malaysia, leaves of *Curcuma longa* are used to wrap fish

before steaming or baking (Larsen *et al*., 1999). Leaves of *Kaempferia galanga* and *C. longa* are ingredients of various types of curries. Some tribal natives in Malaysia flavour their wild meat and fish dishes with leaves of *Elettariopsis slahmong* (Lim, 2003). In Thailand, *E. slahmong* leaves are eaten as salad. Despite their repulsive stinkbug odour, leaves of *E. slahmong* are considered a delicacy. Traditionally, leaves of *E. latiflora* have been used to relieve flatulence, to improve appetite and as an antidote to poisons. In Okinawa, Japan, leaves of *Alpinia zerumbet* are sold as herbal tea, and are commonly used to flavour noodles and wrap rice cakes. The hypotensive, diuretic, and anti-ulcerogenic properties of tea from *A. zerumbet* leaves have been reported (Mpalantinos *et al*., 1998). Leaves of *Etlingera elatior*, mixed with other aromatic herbs, are used by post-partum women for bathing to remove body odour (Ibrahim and Setyowati, 1999). They are also used for cleaning wounds. Leaves of *Kaempferia rotunda* and *K. galanga* are eaten fresh or cooked as vegetables, and used as cosmetic powder and as food flavouring agents (Ibrahim, 1999). In Peninsular Malaysia, boiled leaves of *Hedychium* species are eaten for indigestion (Ibrahim, 2001). Leaves of *Hedychium* species are sometimes eaten with betel nut to ease abdominal pain. In Thailand, boiled leaves of *Hedychium coronarium* are applied to relieve stiff and sore joints. Leaves of *C. mangga* have been reported to possess higher antioxidant activity compared to its rhizomes (Chan *et al*., 2008).

Plants including Zingiberaceae plant species have the ability to synthesis organic compounds that are structurally varied and complex. These compounds are known as secondary metabolites and they have no recognized role in the maintenance of fundamental life processes in the plants that synthesize them. However, they do have an important role in the interaction of the plant with its

environment. They indirectly support the life system of the plant especially act as a defense mechanism towards the pathogens and parasites by producing compounds such as alkaloids, flavonoids and terpenoids. The essential oil produces by some plants acts as insect attractants for pollination purpose or repelling the herbivorous animals. Human beings have been using secondary metabolites as flavouring agents, fragrances, drugs, insecticides, colouring agents and medicines. The production of these compounds is often low (less than 1% dry weight) and depends greatly on the physiological and developmental stage of the plant itself (Oksman-Caldentey and Inze, 2004; Dixon, 2001).

In vitro culture techniques can be used as the alternatives for consistent and sustainable supply of secondary metabolites from plants. In order to produce reasonable amount of secondary metabolites, optimization of the *in vitro* culture conditions have to be well established such as modification on culture nutrients composition, dissolved oxygen content, light intensity, types and volume of culture vessels as well as the suitability of the certain elicitors (Indrayanto, 1997). However, *in vitro* culture technique may not be able to apply successfully to all plant species. This is because production of secondary metabolites in *in vitro* plants has three possibilities. Firstly, the amount of secondary metabolite produced from *in vitro* plants may be higher than that from the mother plant. Secondly, the *in vitro* cultured plants cannot produce specific secondary metabolites that are found in the mother plants or on the other hand the secondary metabolite produced is relatively lesser than the mother plant. Thirdly, secondary metabolites produced from the *in vitro* plants are completely different from those in the mother plant.

However, some studies had been successfully done on *in vitro* shoot cultures in order to produce secondary metabolites using elicitation methods. The total

antioxidant phenolics contents of Oregano (*Origanum vulgare* L) increased by more than 120% in the presence of exogenous proline and under nutritional deficiency compared to normal condition (Lattanzio *et al*., 2009). The production of vindoline, the uncondensed form of vincristine, from 12 days old *Catharanthus roseus in vitro* plantlets were reported to increase 10 times more than the normal condition after supplemented the culture medium with 0.1 µM jasmonate for 12 hours (Hernández-Domínguez *et al*., 2003). (Liu *et al*., 2008)reported that the content of hypericin, pseudohypericin and hyperforin, anti depressant agents, from *Hypericum samsonii in vitro* plantlets was associated with morphological changes due to elicitors.

To date, there is no study reported on the effect of abiotic (pH, light intensity and micronutrient) and biotic (yeast extract and chitosan) factors towards the *in vitro* culture of *C. mangga* plantlets. Thus, this project was carried out with the following objectives:

1. To observe the effect of elicitors towards morphology of *Curcuma mangga*.
2. To determine the suitable elicitors to enhance the antioxidation activity of *in vitro* plantlet extracts of *Curcuma mangga*.
3. To determine the best extract(s) that exhibit the highest antioxidation activity towards DPPH free radical scavenging assay and anti lipid peroxidation assay using Ferric Thiocyanate assay
4. To determine the total phenolics content of extracts and its contribution to antioxidation activity.

CHAPTER TWO

LITERATURE REVIEW

2.1. Zingiberaceae

2.1.1 The Origin and Distribution of Zingiberaceae

The term 'gingers' generally refers to members or species of Zingiberaceae. Zingiberaceae gained its name probably from the fusion of the Arabic word *zanjabil* and Sanskrit word *singabera* (means horn-root) and later was translated as *zingiber* in Latin. Botanically, the name *zingiber* comprises the whole ginger family (Zingiberaceae).

Zingiberaceae encompasses approximately about 1200 species of which about 1000 species were found in tropical Asian region. Malesian region which consists of Indonesia, Malaysia, Singapore, Brunei, the Philippines, East Timor and Papua New Guinea was by far considered as the richest area with 24 genera and about 600 species. That number is an underestimated figure as certain regions such as Borneo and Sumatra are classified as underexplored for the gingers flora (Larsen *et al*., 1999). Many species hence can be discovered in the near future.

Even though the Zingiberaraceae family is well-distributed to all over tropical regions in the world, many species are still inadequately identified taxonomically (Thelaide and Mood., 1997; Sakai and Nagamasu., 1998; Poulsen *et al.*, 1999; Williams *et al.*, 2002). Some genera were newly discovered in the past one decade (Newman, 1995, Mood and Larsen., 1997, Larsen and Mood., 1998, Sakai and Nagamasu., 2000, Kress and Larsen., 2001, Larsen and Jenjitkul., 2001).

The currently accepted classification of Zingiberaceae states that this family comprises of four tribes namely Hedychieae (22 genera), Alpinieae (25 genera),

Zingibereae (one genus) and Globbeae (four genera). This classification is based on both vegetative and floral characteristics. However, in most cases, there is no specific defining character for one particular tribe (Holttum, 1950; Burtt and Smith., 1972; Larsen *et al.*, 1998). Therefore, the taxonomy of Zingiberaceae still remains disputable.

2.1.2 Importance of Zingiberaceae

For decades, traditional folks have used the rhizomes of ginger plants (family Zingiberaceae) as the major source of their remedies and spices. The rhizomes are consumed raw or processed for flavouring food. Major commercially cultivated Zingiberaceae species are *Zingiber officinale* (commonly known as *ginger* in English or *halia* in Bahasa Malaysia), *Curcuma longa* (turmeric or *kunyit*) and *Alpinia galanga* (*galangal*). In many alternative medications such as *Ayurvedic, Jamu,* traditional Chinese medicine (TCM) and traditional Malay medicines, gingers are mostly found as one of the ingredients. Traditionally, the rhizomes of ginger plants are consumed for carminatives during illness or confinement (Larsen *et al*., 1999). Apart from the medicinal values, ginger plants play an important role in many other aspects like culinary, garment dyeing, perfumery and floristry.

Curcuma longa or commonly known as turmeric is the most explored species among entire genus of Curcuma. In *Ayurvedic*, it is commonly used for the treatment of arthritis, anorexia, cough, diabetic wounds, rheumatism, and sinusitis, as well as muscular, hepatic, and biliary disorders (Ammon *et al*., 1992). Scientifically, *C. longa* has been proven to posses anti-hyaluronidase activity (Ramsewak *et al.*, 2000; Ramchandran *et al.*, 2000; Mishra and Gupta, 1997), antioxidant activity (Ramsewak *et al*., 2000, Scartezzini and Speroni, 2000, Ruby *et al*., 1995, Selvan *et al*., 1995,

Anto *et al*., 1994), hepatoprotective activity (Deshpande *et al.*, 1998; Rajshekaran *et al.*, 1998), hypolipidemic activity (Darka, 1999; Deshpande *et al.*, 1996), anti-allergic activity (Yano *et al.*, 2000; Yano *et al.*, 1996), wound-healing activity (Pandya, 1995), anti-microbial activity (Negi *et al*., 1999), anti-fungal activity (Wuthiudomelert *et al.*, 2000; Behura *et al.*, 2000; Apisariyakul *et al.*, 1995), anti-bacterial activity (Rath *et al.*, 1999; Singh *et al.*, 2002), insect-repellent activity (Venugopal and Saju, 1999), anti-ulcer activity (Rafatullah *et al*., 1990), antitumor activity ((Duvoix *et al.*, 2005; Kim *et al.*, 2001; Khar *et al.*, 1999; Kuttan *et al.*, 1985), anti-fertility and anti-spermatic activity (Bhagat and Purohit, 2001), anti-venom activity (Ferreira *et al*., 1992), anti-emetic activity (Deitelhoftt *et al*., 2002) and anti-depressant activity (Yu *et al.*, 2002b; Xu *et al.*, 2005).

Curcuma xanthorrhiza, also known as big turmeric, is one of the important medicinal plants. *C. xanthorrhiza* has been traditionally used to treat stomach diseases, liver disorders, constipation, bloody diarrhoea, dysentery, fevers, haemorrhoids, and skin eruptions (Lin *et al.*, 1996; Yasni *et al.*, 1994). Studies had been conducted to explore its efficacy as antitumor (Itokawa *et al*., 1985), anti hypotriglyceridaemic (Yamazaki *et al*., 1988), anti-inflammatory (Ozaki, 1990), and hepatoprotective agent (Li *et al*., 1995).

Curcuma zedoaria or usually known as *zedoary* is cultivated as a vegetable, spice, and perfumery material in Southeast Asian countries. The rhizome of this plant has been traditionally used as a stimulant, stomachic, carminative, diuretic, anti-diarrhoea, anti-emetic, anti-pyretic, depurator, and also as an ointment for ulcers, wounds, and other skin disorders (Prajapati *et al*., 2003). The chemical constituents of *C. zedoaria* exhibited some potential bioactivities like vasorelaxant, hepatoprotective and inhibitory activity of Nitric Oxide production (Yoshioka *et al*.,

1998). Anti inflammatory activity of *C. zedoaria in vivo* has been reported to be comparable to Indomethacin, a commercially available anti-inflammatory drug (Makabe *et al*., 2006). One of the secondary metabolite named ethyl-*p*-methoxy cinnamate isolated from rhizome of *C. zedoaria* was responsible for the antifungal activity (Gupta *et al.*, 1976; Joshi *et al.*, 1989). The antibacterial activity of *C. zedoaria* extracts and its essential oils against Gram positive and Gram negative bacteria have been reported and found to be due to the presence of mono and sesquiterpenes (Banerjee *et al*., 1978). One recent study confirmed the anti-bacterial and anti-fungal activity of *C. zedoaria* and was found to perform better than *C. malabarica* (Wilson *et al*., 2005). The anti-cancer activity of *C. zedoaria* against human ovarian cancer (OVCAR-3) *in vitro* has been proven and the compounds responsible were the curcuminoids (Syu *et al*., 1998). The polysaccharides of *C. zedoaria* extract showed immuno-enhancer activity by stimulating *in vitro* macrophage activity (Kyung *et al*., 2001)

Curcuma amada, a very famous ginger species in India, has been traditionally used to encounter stomach aches and flatulence (Hussain *et al*., 1992). Scientific studies indicated the potential of *C. amada* as anti-hypercholesterolemia in rabbit (Pachuri and Mukherjee, 1970), anti-fungal (Gupta and Banerjee, 1972), anti-inflammatory *in vivo* in albino rats (Mujumdar *et al*., 2000) and anti-bacterial agents (Chandarana *et al.*, 2005; Policegoudra *et al.*, 2007).

Zingiber officinale or ginger has been reported to possess anti-oxidant activity due to its pungent substance named gingerol as determined by Inhibition of phospholipid peroxidation induced by the Ferric Chloride-ascorbate system (Aeschbach *et al*., 1994). Some other researches reported that gingerol and several other pungent compounds like zingerone and dehydrozingerone of ginger were

responsible for anti-oxidant activity (Kikuzaki and Nakatani, 1993; Kikuzaki *et al.*, 1994; Rajakumar and Rao, 1993, 1994). The aqueous extract of *Z. officinale* was found to inhibit platelet aggregation *in vitro* and that result was correlated with inhibition of synthesis of thromboxane and some prostaglandins (Srivastava, 1984, 1986). Ginger was found to possess an anti-tumour promoting potential as determined by inhibition of phorbol ester-induced inhibition of Epstein–Barr virus EBV's activation in Raji cells (Koshimizu *et al*., 1988). Topical application of ginger extract was reported to protect mouse skin carcinogenesis initiated by 7,12-dimethylbenz[*a*]anthracene (Katiyar *et al*., 1996).

2.1.3 *Curcuma mangga* Val.

C. mangga (Fig 2.1 A) is an annual herbaceous plant that can grow up to 90 – 120 cm in size. It grows in clusters and has pseudostems. The lateral rhizomes are branched from the main rhizomes. The longitudinal section of the rhizomes (Fig 2.1 C) consists of lateral or outer circle which is pale to light yellow in colour and inner circle which is intensively yellow with nodular texture of fibre just like the texture of ginger (*Zingiber officinale*). The rhizomes produce raw mango-like aroma that makes this species distinguish among its close relatives. *C. mangga* rhizomes are freshly eaten as delicacy. In north-eastern India, there is a similar curcuma species that produces fresh mango-like aroma called *C. amada*. The inflorescences (Fig 2.1 B) are emerged among basal leaves, dense, cylindrical, bracts of main axis crowded, small bracteoles, inconspicuous, hidden by bracts. Flowers are yellowish to lime green in colour with short-cylindrical calyx, funnel-form tube corolla, and the lobes are ovate or oblong. The rectangular or plane filaments enclose the anthers in corolla, terminal appendage sometimes present, large lateral staminodes, and concave lip-like

petals. Leaves are eclipse-oblong shape narrowing at the tip, 15 – 95 cm in length and 5 – 23 cm in width, green in colour with purplish shading along the principle vein. *C. mangga* has fibrous rooting system. The roots are mainly generated from the prime rhizome and mature lateral rhizomes (Gusmaini *et al*., 2004). Other species with close relativity to *C. mangga* are *Curcuma xanthoriza, Curcuma aeroginosa, Curcuma zedoaria* and *Curcuma domestica* (Rukmana, 1994).

Curcuma mangga, another Curcuma species with mango-like smell, is traditionally used to relieve fever, stomach ache, general debilities and also for postpartum care especially to aid womb healing (Suhaila *et al*., 1996). Zerumin B, a chemical compound isolated from rhizomes of *C. mangga*, exhibited potential anticancer activity against human breast cancer cells (MCF 7) (Abas *et al*., 2005a). Anti-allergic activity of *C. mangga* was reported to be moderately effective compared to *Kaempferia parviflora* (Tewtrakul and Subhadhirasakul, 2007). The fresh extract of *C. mangga* rhizomes exhibited anti bacterial activity against *Bacillus cereus* and *Staphylococcus aureus* and its essential oils also showed potential anti bacterial activity against *Bacillus cereus*, *Staphylococcus aureus* and *Salmonella thyphi* (Chaisawadi *et al*., 2007).

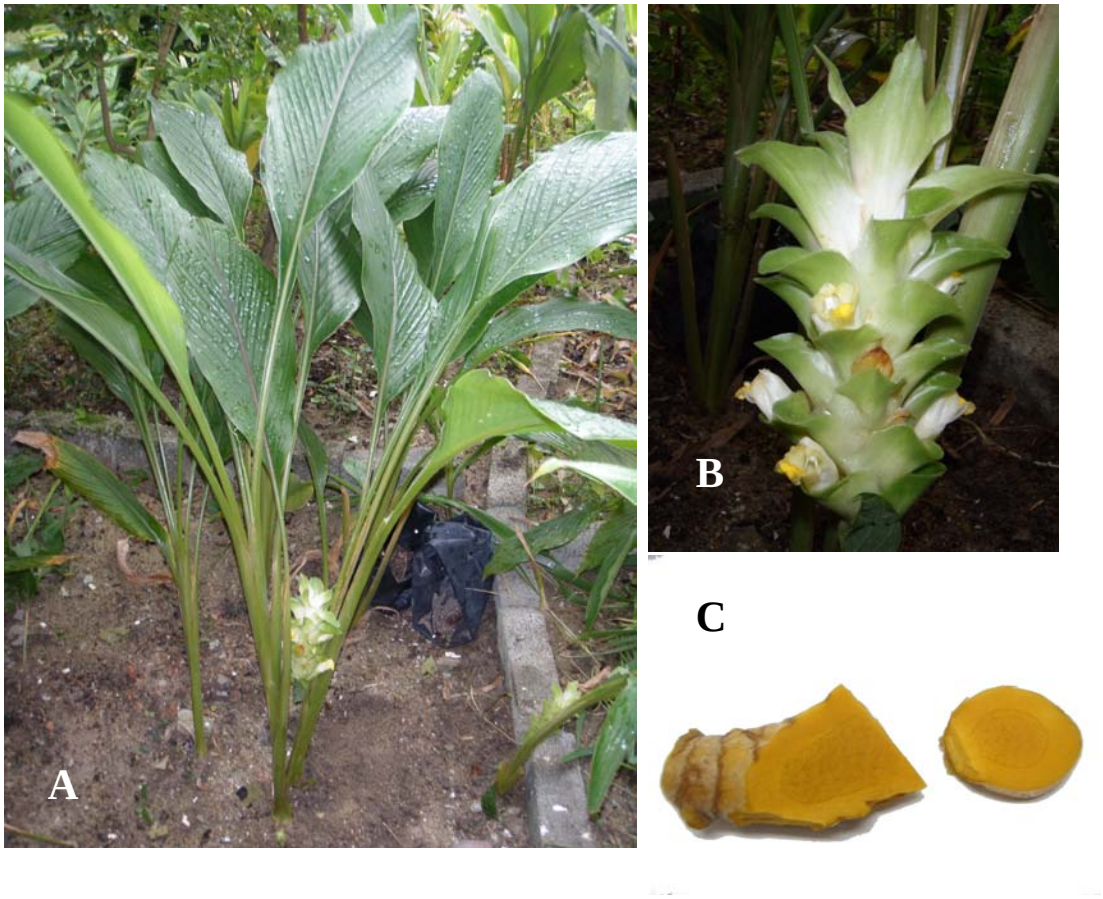

Figure 2.1 Full grown plants of *C. mangga* Val with inflorescence (A), flower (B) and rhizomes (C).

2.2 Plant Tissue Culture Technique

The term plant tissue culture generally refers to the cultivation *in vitro* of all plant parts (single cells, tissues and organs) under aseptic conditions. Plant tissue culture systems are often used as a model system in the study of various physiological, biochemical, genetic and structural problems related to plants. Plant tissue culture techniques also have great prospective by means of vegetatively propagating economically important crops and crops of future potential on a commercial basis (Brown and Thorpe, 1984).

Plant tissue culture technique is divided into two major classifications namely cultures of unorganized tissues and cultures of organized structures. There are four subdivisions under unorganized tissue cultures such as callus (or tissue) cultures, suspension (or cell) cultures, protoplast cultures and anther cultures. There are five subdivisions under organized tissue cultures namely meristem cultures, shoot cultures, node cultures, embryo culture and isolated Root cultures (George, 1993).

Conventional agriculture practice has been facing many obstacles such as seasonal dependant, geographic and year-to-year variations, pest/diseases infestation and recently, climate change. Plant tissue culture technique can be considered as an answer to overcome those obstacles because of two main reasons, firstly, it is done under controlled environment and secondly, it is done in aseptic way. Plant tissue cultures technique is capable of manipulating both organized and unorganized growth of the plant. The organized growth is responsible of maintaining and inducing a defined or complete structure of plant. The unorganized growth is a rare natural phenomenon of plant but it frequently occurs *in vitro* when a piece of plant (explants) is cultured in the medium supplemented with auxins (George *et al*., 2008d). The explants are usually derived from a small part of plant tissue or organ

such as stem tips, leaf, axillary buds, vegetative shoot tips, nodule sections, root sections and reproductive parts such as microspores, megaspores, ovules, embryos, seeds and spores. The explants are inoculated into an aseptic culture vessel containing nutrient medium and incubated under controlled environmental conditions (quality and quantity of illumination, temperature, humidity, etc) to enable them develop to into fully grown plants that commonly refer as plantlets. A basic plant tissue culture medium usually contains various salt solution needed for plant growth and differentiation. In addition, some other components are often added into the culture media mainly for improving growth and differentiation of the *in vitro* plant such as sugar (usually sucrose), vitamins, myo-inositol, and plant growth regulators. Amino acids and other nitrogen sources and 'undefined' supplements such as fruit juices, yeast extracts and casein hydrolysate or coconut water are occasionally added for growth improvement. A solidifying agent added to the medium serves as a matrix to retain water and all of the chemical components of the medium and gradually diffuse the entrapped nutrient to explants. Once the explants develop into plantlets, they will actively absorb the nutrients from the solid medium using roots or their vascular system (George *et al*., 2008a). Theoretically, each of plant cells carries all the genetic information. Plant tissue culture technique works on cell totipotential ability of which it is able to multiply and differentiate into an identical plant as its parent plant (Fowler, 1986).

The term shoot culture is now referred to the cultures started from explants bearing an intact shoot meristem, whose purpose is shoot multiplication by the repeated formation of axillary branches. In this technique, newly formed shoots serve as explants for repeated proliferation; severed shoots are finally rooted to form

plantlets which can be grown *in vivo*. This is the most widely used method of micropropagation.

Shoot cultures are conventionally started from the apex of a lateral or main shoot, up to 20 mm in length, dissected from actively-growing shoots or dormant buds. Larger explants are also sometimes used with advantage. They may consist of larger part of the shoot apex or stem segments bearing one or more lateral bud. Sometimes shoots from other *in vitro* cultures are employed. Large explants also have an advantage over smaller ones for initiating shoot tip cultures in that they survive better in *in vitro* conditions, grow more rapidly and contain more axillary buds.

However, the greater the size of the explant, the more difficult to decontaminate from micro-organisms. Shoot cultures are also frequently started directly from the shoots obtained from meristem tip cultures. Meristem tip or meristem cultures are used for virus elimination. Meristem cultures are initiated from much smaller explants and a single plantlet is usually produced from each explant.

The growth and proliferation of axillary buds/shoots in shoot cultures are usually promoted by incorporating growth regulators (usually cytokinins) into the growth medium. Treatment with plant growth regulators effectively removes the dominance of apical meristems so that more axillary shoots are produced. These shoots are used as miniature cuttings for plant multiplication (George *et al*., 2008c)

2.3 Elicitors

2.3.1 Functions of elicitors

Elicitors are chemicals or biofactors from various sources that can induce physiological changes of the target living organism. In a broad sense, elicitors for a plant refer to chemicals from various sources that can trigger physiological and morphological responses and phytoalexin accumulation. It is commonly known as stress factors and may include abiotic elicitors such as metal ions (Lead, Cadmium, Copper, Zinc, etc), physical factors (temperature, illumination, radiation, acidity, drought, etc) and biotic elicitors from fungi, bacteria, viruses or herbivores, plant cell wall components, as well as chemicals that are released at the attack site by plants upon pathogen or herbivore attack. It is well known that treatment of plants with elicitors, or attack by incompatible pathogens, caused an array of defence reactions, including the accumulation of a range of plant defensive secondary metabolites such as phytoalexins in intact plants or in cell cultures. From a pathogenesis point of view, many elicitors may act as avirulence determinants of a plant genetic system that refers to a general response of gene-for-gene resistance in plant innate immunity, in which plant resistance genes (R genes) confer resistance to pathogens with a matching avirulence (avr) gene by specific recognition events. Elicitors or avirulence determinants must be recognized by plant receptors or R proteins localized to the plasma membrane or the cytoplasm before initiating signaling pathways, which lead to defense reactions such as synthesis of PR (pathogenesis-related proteins), or defense secondary metabolites. Molecular recognition and physical interaction between elicitor signal molecules and specific plant receptors are complex processes but are required for specific elicitor signal transduction. By causing changes in receptor conformation or activation of receptor kinases, elicitors subsequently or

indirectly activate their corresponding effectors, such as ion channels, G-proteins, lipases, and kinases, which then transduce the elicitor signal to downstream defense responses (Zhao *et al*., 2005). From Plant Tissue Culture point of view, the elicitation method is the prototype of plant's natural phenomenon on defending itself toward the pathogen. In this event, the host-pathogen interaction will induce the phytoalexins formation. Phytoalexins, low molecular weight compounds produced by higher plants, play a role as antibiotic to block or suppress the pathogen's invasion (Yoshikawa and Sugimoto, 1993). Elicitors not only induced phytoalexin formation but also non-phytoalexin's secondary metabolites production in callus and cell of Legume (Epperlein *et al*., 1986).

2.3.2 Abiotic elicitors

2.3.2.1 pH of Medium

The relative acidity or alkalinity of a solution is determined by its pH. This is a measure of the hydrogen ion activity (concentration) in solution. The greater the activity of H^+ ions, the more acidic the solution. As pH is defined as the negative logarithm of hydrogen ion activity, acid solutions have low pH values (0-7) and alkaline solutions have high values (7-14). The pH of culture medium must be adjusted in such that it will not disrupt the function of plant cell membranes or the buffered pH of the cytoplasm. Some considerations have to include within these physiologically acceptable limits that it also governs whether salts will remain in a soluble form, influences the uptake of medium ingredients and plant growth regulator additives and affects the gelling efficiency of agar (George *et al*., 2008a). Normally, pH of medium in the range of 5.0 to 6.0 is favourable for *in vitro* growth of explants (Chalwa, 2000). A pH higher than 6.0 gives a moderately hard medium

and a pH below 5.0 does not allow satisfactory gelling of the agar (George and Sherrington, 1984). Most importantly, pH of medium is one of the crucial factors contributing to the growth of plants. A pH range of 5.0 to 6.0 is generally suitable for the *in vitro* growth of explant. A pH higher than 7.0 or lower than 4.5 generally inhibits growth and development (Chalwa, 2000). Culture medium with pH of 5.7 – 5.8 (prior autoclaving) was suitable for maintaining all salts in the soluble form (Murashige and Skoog, 1962). The pH of a medium changes at various stages of preparation and culture. Autoclaving generally causes the pH of medium to drop by 0.3 to 0.5 units (Chalwa, 2000). Besides this, changes also occur over the culture duration due to hydrogen release to and/or from the plant itself. Adjustment of pH was found to be specific for cultivation of a particular plant species (Leifert *et al*., 1992). Some studies indicated that pH changes were not solely because of the previously mentioned factors. The presence of plantlets, callus and cells could contribute to the pH change as well. Agar-based medium was progressively acidified in the presence of a living *Ptilotus exaltatus* explant with an equilibrium occurring at pH 4 (Williams *et al*., 1990). The pH of the medium changed dramatically within 48 hours started from the inoculation of *Cucumis* callus onto the medium (Skirvin *et al*., 1986). Similarly, it was reported that the presence of *Panax ginseng* callus made the medium became more acidic than the media without callus (Sarma *et al*., 1990).

2.3.2.2 Illumination

Light is the prime key factor in the energy supply. Light intensity plays an important role in the biosynthesis of medicinally important metabolites. Light quality also had a significant influence on the growth and morphology of *in* and *ex vitro* plants (Warrington and Michell, 1976; Morgan and Smith, 1981; Smith, 1982;

Tibbitts *et al.*, 1983; Mortensen and Stromme, 1987; Economou and Read, 1987; Agrawal, 1992). The total quantity of light that a plant received during illumination directly affected photosynthesis as well as plant growth and yields (Kim and Kozai, 2000). For instance, the photoregulation of flower pigmentation in *Gerbera hybrida* was by gene expression of Chalcone synthase (CHS) and Dihydroflavonol-4-reductase (DFR). The expression was inhibited when plants were grown in the dark and it was found that, blue light promoted gene expression of CHS and DFR, and red light enhanced the gene expression of CHS (Meng *et al*., 2004). The level of the anti prostatic-cancer compound, an alkaloid metabolite from tree of joy (*Camphotheca accuminata*) called camphotecin, increased with decreasing light intensity (Leland and Kaufman, 1999).

Many lighting systems that effectively used electrical energy in the multiplication of horticultural plants have been studied intensively such as fluorescent, incandescent, luminescent (Sodium high pressure) lighting systems, and recently, light-emitting diode (LED) lighting source. Tissue cultured plants are almost invariably grown under fluorescent illumination (Collin *et al*., 1988), especially under cool white fluorescent lamps with a high proportion of its output in the blue and red regions (Hart, 1988).

2.3.2.3 Micronutrients

Micronutrients are essential trace elements required for plant growth. Iron (Fe), Manganese (Mn), Zinc (Zn), Boron (B), Copper (Cu), Cobalt (Co) and Molybdenum (Mo) are the essential metal ions that plants need in a trace amount for metabolism and physiological importance. At least five of these metals are necessary for chlorophyll synthesis and chloroplast function (Sundqvist *et al*., 1980). Artificial

medium usually contains Mn, Co, B, Mo, Fe and Zn as micronutrients. However, nickel and aluminium are often found in the some modified formula. Most of these nutrients play a role as enzyme cofactors.

In plants, iron is primarily used in the chloroplast, mitochondria and peroxisoms for oxidation and reduction purposes. The element is required for the formation of amino laevulinic acid and protoporphyrinogen (both of them are precursors of chlorophyll) and deficiency leads to chlorosis. Iron is also a component of ferredoxin proteins which function as electron carriers in photosynthesis. Plant absorbs iron from media in ferrous (Fe^{2+}) or ferric (Fe^{3+}) ion's form. Ferrous sulphate or ferric citrate or tartrate supplemented into the culture media was found to be inappropriate. It always precipitated and hence was not fully available to the plant cultures. The addition of iron in chelate form with EDTA (ethylene-diamine-tetraacetic acid) was found to be very effective on keeping the solubility of iron in solution (Jacobson, 1951; Weinstein *et al.*, 1951). Further study indicated that iron in chelated form was less toxic and applicable for *in vitro* cultures of isolated tomato roots over a wide range of pH (Street and McGregor, 1952). Another study indicated that the use of Fe-EDTA in White (1945) medium had rapidly increased the growth of several callus rather than in the medium supplemented with pure compound of iron (Klein and Manos, 1960). Skoog and co-workers started to use EDTA in media for tobacco callus cultures in 1956 and incorporated their finding in MS medium (Murashige and Skoog, 1962). Deficiency of iron in *in vitro* plant cells was found to decrease the initial growth of *Acer pseudoplatan* L cells. In the absence of iron, the cell number doubling time was much longer (approximately 100 hours) as compared to sycamore cells grown in liquid medium containing iron (approximately 48 hours). Besides this, iron

deprivation also limited the maximum density of cells produced (Pascal and Douce, 1993). The addition of Iron in medium ten folds more than normal MS iron concentration increased the production of Betalains, a water-soluble food colorant, derived from *Beta vulgaris* by double after three days inoculation (Savitha *et al*., 2006).

Copper (Cu) is a cofactor in a variety of enzymes and electron transfer catalysts, even though plants normally contains only a few parts per million (ppm) of it (George, 1993). The effect of different concentrations of copper was different in various culture systems. Copper deficiency was reported to cause a reduction of cytochrome c oxidase in the mitocondria in cell culture of *A. pseudoplatanus* L (Bligny and Douce, 1977). The effect of doubled dose copper was reported to increase the production of benzophenanthridine alkaloid and dihydrosanguinarine (which has antibacterial activities) by four times on cell suspension culture of *Papaver bramarun* (Lecky *et al*., 1992). However, the yield was only doubled when the copper concentration was increased four folds. Study on *P. ginseng* indicated that the *in vitro* roots of *P. ginseng* accumulated copper in a concentration-dependent and duration-dependent manner. Roots treated with 50 μM copper showed 52 % and 89 % growth inhibition after 20 and 40 days of culture respectively. Saponin synthesis was stimulated at a copper concentration of between 5 and 25 μM but decreased at 50 μM copper. The presence of 50 μM copper in the medium also found to induce oxidative stress in the roots (Ali *et al*., 2006a).

The role of boron (B) in plant biochemistry and physiology is still largely unknown. It is required for the metabolism of phenolic acids and for lignin biosynthesis and necessary for the maintenance of meristematic activity. Most probably it is involved in the synthesis of N-bases (uracil, a component of RNA

synthesis, in particular) (Mengel and Kirkby, 1982). It is also probably involved in the maintenance of membrane structure and function by stabilizing natural chelates which are important in wall and membrane structure and function (Pollard *et al.*, 1977; Clarkson and Hanson, 1980). A study reported that the cultures of *Chenopodium album* L cultured in boron-deficient medium were still capable of growing and producing more enlarged and detached cells (Fleischer *et al*., 1998). Cell death occurred as early as 24 hours following boron deprivation in *Rosa damascena* Mill cv Gloide de Guilan (rose) cell suspension culture. Parallel with it was an increasing amount of phenolics in the medium that indicated a loss of membrane integrity (Dordas and Brown, 2005). In suspension cultures of tobacco, cell propagation ceased immediately and the cells died when the culture media were deprived of boron (Kobayashi *et al*., 2004). Tobacco (*Nicotiana tabacum* L) cultivated in free boron medium increased the polyamines content (putrescine, spermidine and spermine), aliphatic amines that involved in cell proliferation and differentiation in vascular plant, in both of leaves and roots of the plants.

Molybdenum is a component of several enzymes in plants such as nitrate reductase and nitrogenise. It is involved in the conversion of nitrate ions to ammonium, the form required by plants, which is the first step to the formation of proteins and other essential components such as enzymes and chlorophyll. Tissue and organs presented with NO_3^- in a molybdenum-deficient medium exhibited the symptoms of nitrate toxicity because the ion is not reduced to ammonia. Molybdenum is normally introduced in cultures media as sodium molybdate at concentration up to 1 µM. Considerably high concentration of molybdenum was found in Abou-Mandour medium and Asahira and Kano medium without side effect

to the cultured plant. However, pine cell suspension cultures treated with 50 µM of Molybdenum were reported to be injured (Teasdale, 1987)

Zinc is a cofactor for several enzymes such as anhydrases, dehydrogenases, oxidases and peroxidases and plays an important role in regulating the nitrogen metabolism, cell multiplication, photosynthesis and auxin synthesis in plants. Zinc deficient plants suffer from reduced enzyme activities followed by protein deficiency and chlorophyll synthesis failure. The common symptoms of molybdate- and zinc deficient plants are decreasing chlorophyll content and poorly develop chloroplast. There was a close relationship between the zinc nutrition of plants and their auxin content (Skoog, 1940). It was suggested that zinc was a component of an enzyme concerned with the synthesis of the IAA precursor, tryptophan (Tsui, 1948). The importance of Zn for tryptophan synthesis was especially noticeable in crown gall callus which normally produced sufficient endogenous auxin to maintain growth on a medium without synthetic auxins, but which becomes auxin-deficient and ceased to grow in the absence of Zn (Klein *et al*., 1962). The concentration of Zn^{2+} added to cultured media has varied widely, and has ranged from 0.1-70 µM, which seems to indicate that an excess of Zn^{2+} ions is not toxic (George, 1993). The betalains, natural food colorant, produced from the hairy roots of *Beta vulgaris* cultivated in liquid medium supplemented with 20 folds of normal concentration of zinc in control medium increased by 74.3% after three days of culture (Savitha *et al*., 2006).

Manganese (Mn) has similar chemical properties to Mg^{2+} and is most likely able to replace magnesium in some enzyme systems (Hewitt, 1948). However, there are normally 50 to 100 folds more of Mg^{2+} than Mn^{2+} within plant tissues and so it is unlikely that there is a frequent substitution between the uses of the two elements. The most probable role for these elements were in the structure of metalloproteins

involved in respiration and photosynthesis (Clarkson and Hanson, 1980). Manganese is crucial for the activity of several enzymes such as decarboxylases, dehydrogenases, kinases, oxidases and super oxides dismutase enzymes. In tissue culture practice, exclusion of Mn ions from Doerschug and Miller (1967) medium was found to reduce the number of buds initiated on lettuce cotyledons. A high concentration of Mn could compensate the lack of molybdenum in the growth of excised tomato roots (Hannay and Street, 1954). The addition of ten folds normal concentration of Mn in medium enhanced 85.6% of betalains production from *Beta vulgaris* hairy root cultures as compared to the control at seven days of culture (Savitha *et al*., 2006).

Cobalt (Co) is the metal component of Vitamin B_{12} which is responsible in nucleic acid synthesis (Fries, 1962). Murashige and Skoog (1962) included Co in the medium formula because it had been shown to be important for lower plants (Holm-Hansen *et al*., 1965) and it might have a significance role in regulating morphogenesis in higher plants (Miller, 1954; Salisbury, 1959). But, the callus culture of tobacco showed no stimulatory growth in the presence of cobalt chloride in the medium at several concentrations from 0.1 µM and above. At concentrations 80.0 and 160 µM, the cobalt was toxic for tobacco callus. *Pinus* cell suspension cultures did not require cobalt (Teasdale, 1987). The benefit of adding cobalt in the media was derived from the fact that it had a protective action against metal chelate toxicity and it was able to inhibit oxidative reactions catalysed by ions of copper and iron (Albert, 1958). The addition of 20 to 30 folds normal concentration of Co had been reported to inhibit about 50% of betalains production from hairy root cultures of *Beta vulgaris* L at 13 days of culture (Savitha *et al*., 2006).

In conclusion, essential metal ions (micronutrients) influence metabolism pathways which lead to the growth sustainability and development of plant. The metabolism will produce metabolites, both primary and secondary, as the end products. Being exposed to the high concentration of these metal ions could activate some defence mechanisms of plant, such as complex transport networking, chelation and sequestration process, to overcome the excessive amount of metal ions (Halloran and Cullota, 2000; Clemens, 2001). However, when the concentration of those heavy metal ions exceed plant heavy metal tolerance, it could cause intoxicate and detrimental effects to the plants (Gasic and Korban, 2006). Phytochelatins, chemically known as γ-$_{L}$-glutamyl-$_{L}$-cysteinyl-$_{n}$-glycine, are proteins synthesized by plants as a response to metal intoxication. Phytochelatins will form complex molecule with metal ions which is not toxic to the plants. Phytochelatins are produced in small amount in normal cultivation condition of plants. They are actively synthesized under heavy metal stress condition (Kägi, 1993; Grill *et al.*, 1989). The activation of phytochelatins formation may also indirectly activate other biosynthesis pathways which may result in secondary metabolites production.

2.3.3 Biotic elicitors

2.3.3.1 Yeast extract

Yeast extract (YE) had been reported as an additional nutrient for tissue culture medium. It played role an alternative source of amino acids and vitamins, especially inositol and thiamine (Vitamin B_1). But, nowadays YE is used less as a component of culture media (George *et al*., 2008a). The function of YE to promote growth becomes significantly importance in culture medium supplemented with only micro and macronutrients (George *et al*., 2008a). The percentage of amino acids in

YE was found to be considerably high (7% of amino nitrogen) (Nickell and Maretzki, 1969; Thom *et al.*, 1981).

Yeast extract is usually added to culture media in concentration of 0.1-1 g/l. However, some media could contain as high as 5, 10 and 20 g/l (Morrel and Murrel, 1964). The addition of 125-5000 mgL^{-1} YE to MS medium totally inhibited the growth of green callus of five different plant species namely *Daucus carota, Lactuca sativa, Chicorium endivia, Lycoperiscon esculentum* and *Petroselinum crispum* whereas small quantities of YE (125 mgL^{-1}) added to Vasil and Hilderbrandt (1966) THS medium, which contains 40% lesser NO_3^- and NH_4^+ ions and did not contain nicotinic acid and pyridoxine as compared to MS medium, induced more significant growth of carrot, endive and lettuce callus compared to MS medium. It indicated that YE promoted the callus growth of *Daucus carota, Lactuca sativa, Chicorium endivia* in low concentration of nitrogens and absence of vitamins (Vassil and Hilderbrandt, 1966).

Yeast extract has been exhibited to possess some unusual properties which may relate to its amino acid content. It elicits phytoalexin accumulation in several plant species. The formation of narengin in *Glycyrrhiza echinata* suspension cultures was increased in the presence of YE that stimulated chalcone synthase activity (Ayabe *et al*., 1988). The production rosmarinic acids and lithosphermic acid B were increased by the addition of YE in *Salvia miltiorrhiza* hairy root cultures (Chen *et al*., 2001).

2.3.3.2 Chitosan

Chitosans (polycationic *b*-1,4-linked-*d*-glucosamine polymers) are polysaccharides produced from chitin, for instance from crab shells and shrimp